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A gene with major phenotypic effects as a target for selection vs. homogenizing gene flow

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Abstract

Genes with major phenotypic effects facilitate quantifying the contribution of genetic vs. plastic effects to adaptive divergence. A classical example is Ectodysplasin (*Eda*), the major gene controlling lateral plate phenotype in three-spined stickleback. Completely plated marine stickleback populations evolved repeatedly towards low-plated freshwater populations, representing a prime example of parallel evolution by natural selection. However, many populations remain polymorphic for lateral plate number. Possible explanations for this polymorphism include relaxation of selection, disruptive selection or a balance between divergent selection and gene flow. We investigated 15 polymorphic stickleback populations from brackish and freshwater habitats in coastal North-western Europe. At each site, we tracked changes in allele frequency at the *Eda* gene between subadults in fall, adults in spring and juveniles in summer. *Eda* genotypes were also compared for body size and reproductive investment. We observed a fitness advantage for the *Eda* allele for the low morph in freshwater and for the allele for the complete morph in brackish water. Despite these results, the differentiation at the *Eda* gene was poorly correlated with habitat characteristics. Neutral population structure was the best predictor of spatial variation in lateral plate number, suggestive of a substantial effect of gene flow. A meta-analysis revealed that the signature of selection at *Eda* was weak compared to similar studies in stickleback. We conclude that a balance between divergent selection and gene flow can maintain stickleback populations polymorphic for lateral plate number and that ecologically relevant genes may not always contribute much to local adaptation, even when targeted by selection.

Keywords: dispersal, fitness, migration, polymorphism, reproduction, threespine

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Introduction

Both selective processes, such as natural selection, and selectively neutral processes, such as random gene flow and genetic drift, shape natural diversity (Kimura 1983; Endler 1986; Schluter 2000; Edelaar & Bolnick 2012). Divergent selection is thought to enhance adaptive divergence between populations from different environments, while high gene flow is expected to have a constraining effect on this differentiation. Hence, adaptive divergence proceeds as a function of the balance between both forces. This has been suggested by theoretical work (Levene 1953; Endler 1977; Slatkin 1985; García-Ramos & Kirkpatrick 1997; Hendry *et al.* 2001) as well as empirical studies (reviewed in Räsänen & Hendry 2008; Pinho & Hey 2010). Most of these studies have been focusing on adaptive divergence at the

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phenotypic level, because selection acts on phenotypes regardless of their genetic basis. However, the evolutionary response to selection is determined by the underlying genetic architecture of the phenotype, as well as by the strength of gene flow which can counteract frequency changes for the genes that matter for the phenotype (Lenormand 2002). The genetic level thus enhances our understanding of adaptive divergence. It makes it feasible to assess the contribution to adaptive divergence of selection favouring one allele over another, as well as the homogenizing effect of gene flow.

Including the genetic level in studies of adaptive divergence may be accomplished by a combination of field studies describing patterns of adaptive divergence, genomic studies pinpointing to the genetic basis of the traits involved and experiments identifying the fitness consequences arising from the phenotypic effects of specific alleles in a simplified ecological context. Considerable progress in each aspect has been made in the study of the evolution of lateral armour plates in three-spined sticklebacks (*Gasterosteus aculeatus* L., Gasterosteidae, Teleostei; reviewed in Barrett 2010). Lateral plate number represents an important ecological trait with a relatively simple genetic basis. Plate variation has been shown to be controlled by Ectodysplasin (*Eda*), a major effect gene, along with several minor effect genes (Colosimo *et al.* 2005). Two alleles, Eda^C and Eda^L, account for complete and low plate number, respectively. Completely plated, ancestral sticklebacks inhabit marine and estuarine habitats throughout the Northern Hemisphere. Multiple freshwater populations, characterized by reduced plate number, have evolved postglacially in parallel in a relatively short period of 10 000–16 000 years (Bell 2001; Raeymaekers *et al.* 2005). Plate variation has been attributed to a number of abiotic and biotic conditions, including large-scale climatic effects, salinity and calcium levels, variation in water flow and predation pressure (Barrett 2010).

A number of field experiments have started to explore the fitness consequences of allelic variation at *Eda*, pinpointing to some of the functional mechanisms responsible for the evolution of reduced armour in freshwater populations (Barrett *et al.* 2008, 2009; Marchinko 2009; Zeller *et al.* 2012a). For instance, there is evidence that in freshwater, sticklebacks carrying the Eda^L allele have a growth advantage leading to higher survival rates and reproductive output (Barrett *et al.* 2008). This hypothesis was inspired by evidence for differences in length growth between plate morphs (Marchinko & Schluter 2007). Barrett *et al.* (2008) initiated their experiment by introducing adult marine sticklebacks heterozygous at the *Eda* locus to four freshwater ponds. These fish produced juveniles of which length growth and *Eda* allele and genotype frequencies were tracked year round. After one generation, they observed a net increase in the Eda^L frequency. From a set of well-documented introductions of completely plated stickleback in freshwater ponds and lakes, it is known that populations can evolve low plate number in less than twenty years, which is less than or equal to 20 generations (Kristjansson *et al.* 2002; Bell *et al.* 2004; Le Rouzic *et al.* 2011; Bell & Aguirre 2013). This implies that the fitness advantage of the Eda^L allele in freshwater may be considerable.

Despite these results, selection on the *Eda* locus and the number of lateral plates in nature seems variable and more complex than in semi-natural ponds or isolated lakes. For instance, Zeller *et al.* (2012b) observed disruptive rather than divergent selection at the *Eda* locus in a freshwater stream, while no evidence for selection on *Eda* was found in a nearby freshwater pond. In addition, stickleback habitats such as the open sea, estuaries, lagoons, streams and lakes are often well connected. Although there is sometimes evidence for reproductive isolation even between adjacent marine and freshwater populations (Hagen 1967; Jones *et al.* 2006; Bell *et al.* 2010), high gene flow between such environments might occur as well, weakening the response to selection (Baumgartner & Bell 1984; Bell 2001; McKinnon & Rundle 2002; Hendry *et al.* 2009). Clearly, it is important to understand the effect of variety in selection conditions and gene flow on lateral plate divergence. In fact, although the reduction in body armour in freshwater populations compared to marine populations is very common across the stickleback's distribution range, it is not the only pattern observed. Several freshwater populations remain completely plated or evolve only weak armour reduction (Hagen & Gilbertson 1972; Hagen & Moodie 1982; Baumgartner & Bell 1984; Banbura 1994; Klepaker 1995; Bell 2001; McCairns & Bernatchez 2008, 2012; Berner *et al.* 2010; Lucek *et al.* 2010; Leinonen *et al.* 2012; Moser *et al.* 2012), or might even show reverse evolution for plate number (Kitano *et al.* 2008). Likewise, marine and estuarine populations may be highly variable in plate number as well (Banbura 1994; Klepaker 1996; Raeymaekers *et al.* 2007). Overall, there is a bias in the literature towards several prominent studies on stickleback investigating sharp contrast divergence between completely plated marine and low-plated resident freshwater populations (e.g. Cresko *et al.* 2004; Colosimo *et al.* 2005; Barrett *et al.* 2008; Schluter & Conte 2009; Jones *et al.* 2012), mostly from Northern Europe and the Pacific Coast of North America, while vast regions in Western and Central Europe and the Atlantic Coast of North America show weaker contrasts (e.g. Münzing 1963; Hagen & Moodie 1982; Raeymaekers *et al.* 2007; McCairns & Bernatchez 2008; Lucek *et al.* 2010).

In this study, we aim to explore the effects of variation in selection conditions and gene flow on the dynamics of the *Eda* locus in natural stickleback populations. An excellent system in this respect can be found in the estuaries and coastal lowlands of North-western Europe (Fig. 1; Heuts 1947; Raeymaekers *et al.* 2005, 2007, 2012). Populations in this area are all polymorphic for plate number. We here start from the observation that these populations occur in freshwater as well as brackish water. A number of mechanisms, including

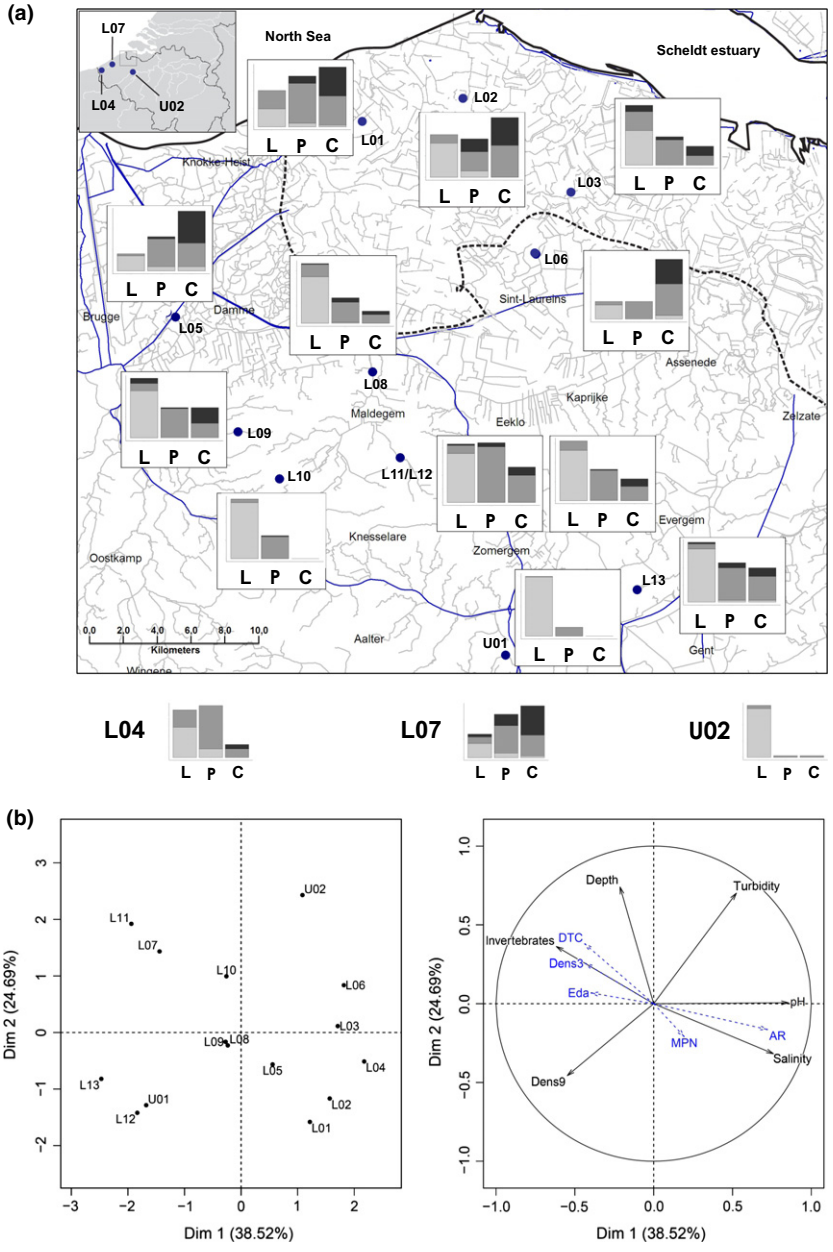

Fig. 1 (a) Locations and characteristics of 15 polymorphic stickleback populations from the Belgian-Dutch lowlands, investigated between spring 2008 and fall 2010. The bar charts show the relative plate morph frequencies in every site (L, low-plated individuals; P, partially plated individuals; C, completely plated individuals). The proportion of homozygotes for the Eda^{L} allele (LL), heterozygotes (CL) and homozygotes for the Eda^{C} allele (CC) is shaded in light grey, dark grey and black, respectively. (b) Individual factors map (left) and variable factors map (right) of a principal component analysis on habitat characteristics of the 15 sampling sites. Variables labelled in black (full arrows) were used to calculate the principal components; variables in blue (dotted arrows) were included as supplementary variables. Dens3: density of three-spined stickleback. Dens9: density of nine-spined stickleback. Eda: frequency of the *Eda* allele for the low morph. MPN: mean plate number. Invertebrates: density of macro-invertebrate predators. DTC, distance to the coast. AR, allelic richness at neutral markers. Population codes as in Table 1.

time since divergence, genetic constraints, balancing selection (including overdominance and negative frequency dependent selection), relaxation of selection, environmental heterogeneity and a balance between divergent selection and gene flow, may explain why populations remain polymorphic for ecologically relevant traits (Endler 1973, 1977; Forsman & Shine 1995; Joron *et al.* 1999; Merilaita 2001; Brooks 2002; Hoekstra *et al.* 2004; Larmuseau *et al.* 2010). Here, we hypothesize that a balance between divergent selection and gene flow maintains the polymorphism for plate number. Indeed, divergent selection pressures may be present (e.g. related to salinity), but adaptive divergence may be hampered by subadaptive or maladaptive gene flow. Considerable gene flow among local stickleback populations seems likely, because part of the study area is well connected through a dense network of water bodies (Fig. 1). Moreover, some populations might be anadromous, facilitating gene flow upon spawning migrations between brackish and freshwater in spring (Tudorache *et al.* 2007). The combined effect of divergent selection vs. homogenizing gene flow might as well shape phenotypic distributions at the landscape level (Endler 1973, 1977; Bell & Richkind 1981; Bell 1982; Baumgartner 1986; Moore *et al.* 2007).

To test the selection – gene flow scenario, we selected 15 polymorphic populations from a range of brackish and freshwater habitats and investigated the dynamics of the *Eda* gene from spring 2008 until fall 2010. We first tested for environment-fitness correlations (indicative for selection; Endler 1986) by comparing populations from different salinities for changes in *Eda* allele frequencies between life stages and by comparing *Eda* genotypes for body size, reproductive investment and reproductive success. We then quantified gene flow between all populations using microsatellite markers. The strength of selection on the *Eda* gene relative to the strength of gene flow was compared with standard tests for genomic signatures of selection. Finally, we performed a meta-analysis to compare the relative contribution of divergent selection vs. gene flow to variation in plate number and allelic variation at the *Eda* gene across various spatial scales.

Material and methods

Study area

Three-spined sticklebacks from the coastal lowlands of Belgium and the Netherlands are either anadromous or landlocked and live in ponds, ditches, small streams, estuaries or polder creeks (Wootton 1976; Raeymaekers *et al.* 2005, 2007). The area contains diked brackish and freshwater habitats of Holocene origin with varying connectivity to adjacent estuaries and the open sea. Habitats are usually shallow (<1.5 m). The water current is slow to stagnant. Three-spined and nine-spined sticklebacks (*Pungitius pungitius* L.) dominate the fish community. The three-spined stickleback populations contain varying percentages of the three plate morphs as defined by Ziuganov (1983), that is the low-plated morph (10 or fewer plates), the partially plated morph (11–20 plates) and the completely plated morph (more than 20 plates). Populations that are 100% low plated do occur outside the study area, in rivers and streams further inland (Raeymaekers *et al.* 2008, 2009).

Field sampling and morphology

We selected 15 sites, including six brackish creeks, two freshwater ponds, four freshwater ditches and three freshwater streams (Table 1; Fig. 1). Each of the sites was visited twice in spring, twice in summer and twice in fall (spring 2008, fall 2008, spring 2009, summer 2009, summer 2010 and fall 2010). Droughts in the summer of 2010 and inundations in the fall of 2010 prohibited sampling some of the sites. Fieldwork started with monitoring the water for temperature (°C), pH, conductivity (μS/cm; for analyses converted to salinity in psu) and oxygen (mg/L) using a Hach field-monitoring unit (Hach, Loveland, Co, USA). An index for the turbidity of the water was obtained with a Sneller tube. The water depth was calculated as the median depth (cm) of five equidistant points along a stretch of 100 m of the water body. A single person (JAMR) then sampled three-spined and nine-spined sticklebacks by progressively dipnetting along the same stretch of 100 m with approximately one dip per metre, each time using the same hand net. This method enabled us to estimate the density of three-spined and nine-spined sticklebacks as the number of individuals per metre. While fishing, the total density of macro-invertebrate predators of stickleback was determined as well by counting all backswimmers (*Notonecta glauca*), dragonfly larvae (*Anax* sp. and *Aeschna* sp.) and great diving beetles (*Dytiscus marginalis*). Sticklebacks were immediately anaesthetized and flash frozen in dry ice after capture. In spring 2009, an additional sampling was performed to search for predatory fishes such as perch, eel and pike. We here used electrofishing in freshwater and a seine net in brackish water. The sticklebacks captured on this occasion were excluded from all analyses.

In the laboratory, the sticklebacks were thawed on ice, measured (standard length (SL); ±0.1 cm), weighed (±0.01 g), photographed and fin-clipped. Spring-caught samples were assessed visually for any external signs of reproductive investment. Reproductive males were identified based on the characteristic red coloration of

Table 1 Characteristics and sample sizes of 15 lowland populations of three-spined stickleback from Belgium and the Netherlands, sampled between spring 2008 and fall 2010

Site	Habitat	DTC (km)	AR	F_{ST}	MPN	Eda^L	Salinity (psu)	Dens3 (ind/m)	Dens9 (ind/m)	Depth (cm)	pH	Turbidity	Inv (ind/m)	*N* (plate number)	*N* (genetics)
L01	Creek	3.94	10.86	0.01	17.40	0.44	2.04	0.15	0.52	33.06	7.89	−22.25	0.04	81	193
L02	Creek	4.30	10.74	0.02	16.81	0.51	1.83	0.14	0.30	33.19	7.78	−21.25	0.01	67	218
L03	Creek	6.95	9.80	0.05	11.80	0.47	0.81	0.11	0.23	36.08	7.91	−16.25	0.01	35	77
L04	Creek	7.50	10.71	0.02	12.64	0.62	2.38	0.39	0.19	n.a.	7.90	n.a.	0.00	26	83
L05	Creek	10.90	11.08	0.01	19.27	0.42	1.04	0.23	0.17	49.69	7.69	−25.46	0.00	56	212
L06	Creek	11.14	10.77	0.01	18.85	0.47	2.35	0.06	0.08	73.54	7.69	−20.00	0.00	27	72
L07	Ditch	11.52	10.52	0.02	17.55	0.44	0.42	0.44	0.14	59.17	7.43	−21.67	0.15	128	307
L08	Stream	17.48	8.90	0.05	10.01	0.73	0.39	0.84	0.14	51.46	7.53	−25.42	0.00	113	233
L09	Ditch	18.30	7.15	0.11	13.46	0.63	0.52	0.14	0.10	39.53	7.42	−22.75	0.03	116	137
L10	Pond	21.75	3.18	0.51	8.83	0.84	0.32	0.34	0.19	63.60	7.45	−19.67	0.01	93	181
L11	Pond	22.84	6.31	0.15	13.68	0.58	0.18	1.05	0.55	71.65	7.50	−17.00	0.16	113	169
L12	Stream	22.84	7.86	0.08	12.66	0.74	0.32	0.72	0.35	40.25	7.17	−28.50	0.01	122	286
L13	Ditch	27.73	6.03	0.17	13.60	0.74	0.52	0.96	0.65	66.25	7.44	−30.33	0.08	79	206
U01	Ditch	36.20	7.07	0.12	7.02	0.93	0.52	1.86	0.60	56.63	7.49	−29.25	0.03	119	167
U02	Stream	51.25	7.49	0.10	5.85	0.89	0.46	2.01	0.00	82.75	7.78	−17.00	0.01	34	86

DTC, distance to the coast; AR, allelic richness at neutral markers; F_{ST}, population-specific F_{ST} at neutral markers; MPN, mean plate number; Eda^L, frequency of the *Eda* allele for the low morph; Dens3, density of three-spined stickleback; Dens9, density of nine-spined stickleback; Inv, density of macro-invertebrate predators; *N*, sample size for plate number and population genetic analyses.

the throat, while reproductive females were identified based on the development of eggs. Individuals smaller than 25 mm SL were preserved in 100% ethanol, while larger individuals were stored on a 4% formalin solution. After 2 months, a subsample of the formalin-stored fish was rinsed with water for 72 h, bleached for 4 h (1% KOH bleach solution) and stained with alizarin red S to facilitate plate counts and plate morph registration (Taylor & Van Dyke 1985). After staining, the number of lateral plates on the left side was determined. Based on this number, specimens were categorized as low-plated, partially plated or completely plated, as defined above. The presence of a keel, a small modification of the caudal lateral plates, was noted, but not included in the plate count.

DNA extraction and genotyping

Genomic DNA was extracted from fin clips using the Nucleospin 96 Tissue DNA Extraction kit (Macherey-Nagel) according the manufacturer's protocol. Allelic variation was assessed at 15 microsatellite loci and one locus (*STN380*) linked to the *Eda* gene (Peichel *et al.* 2001; Colosimo *et al.* 2005; Mäkinen *et al.* 2008) divided over 2 multiplex reactions. Multiplex 1 contained loci *Gaest66*, *STN26*, *STN30*, *STN130*, *STN173*, *STN174*, *STN185*, *STN196* and *STN380*, while multiplex 2 contained loci *Gaest4*, *STN3*, *STN23*, *STN52*, *STN61*, *STN148* and *STN219*. All loci were amplified with the Qiagen® Multiplex PCR Kit (Qiagen, Venlo, the Netherlands). The 10 μL PCR cocktail contained 1–100 ng genomic DNA, 0.05 μM (*STN26*, *STN130*, *STN173*), 0.1 μM (*Gaest66*, *STN3*, *STN30*, *STN174*, *STN185*, *STN196* and *STN219*) or 0.2 μM (*Gaest4*, *STN23*, *STN52, STN61*, *STN148* and *STN380*) forward and reverse primer, 1 × Qiagen multiplex PCR master mix (3 mM $MgCl_2$) and RNase-free water. The reaction consisted of an initial activation step of 15 min at 95 °C, followed by 26 cycles of 30 s at 95 °C, 90 s at 53 °C and 60 s at 72 °C. A final elongation step of 30 min at 60 °C was performed. PCR products were visualized on an ABI3130 Avant Genetic analyzer (Applied Biosystems, Foster City, CA, USA). Allele sizes were determined by means of an internal GeneScan 500-LIZ size standard, and genotypes were obtained using GENEMAPPER 4.0 (Applied Biosystems). Genotypes were checked for scoring errors using MICRO-CHECKER 2.3 (van Oosterhout *et al.* 2004).

Data analysis

Analyses aimed at describing (i) habitat characteristics, (ii) population genetic structure and (iii) the variation in lateral plate number and at the *Eda* gene. We also investigated (iv) signatures of selection on plate number and the *Eda* gene within populations, and (v) the gene flow–selection balance for plate number and *Eda* at the landscape level. Total sample sizes for plate number and population genetic analysis are provided in Table 1. A schematic overview of the analyses together with sample sizes per *Eda* genotype per season is provided in Table S1 (Supporting information). Unless mentioned otherwise, analyses were performed in R (R Core Team 2012).

Habitat structure. Habitat characteristics included salinity, pH, water depth, the density of nine-spined stickleback, the density of macro-invertebrate predators and turbidity. We first investigated the temporal stability of the biotic and abiotic environment across sites over the entire period (2008–2010). We then performed a principal component analysis on mean values of each of these variables. The first and second principal components were plotted to detect clusters of sites with similar habitat characteristics and to investigate how habitats change with distance to the coast (DTC, measured as the crow flies).

Population genetic structure. First, genetic diversity was calculated as the observed heterozygosity (H_O) and as allelic richness (AR; i.e. the number of alleles standardized for sample size and averaged over loci), as implemented in the HIERFSTAT package in R. These metrics were used to test whether or not genetic diversity declines with distance to the coast. Temporal replicates (2008–2010; overview in Table S1, Supporting information) were pooled by site for this purpose. Second, overall and pairwise population differentiation was quantified with the ADEGENET package in R using the standardized allelic variance F_{ST}. Pairwise F_{ST} values were used to visualize population structure with a two-dimensional classical multidimensional scaling (CMDS) plot with the function cmdscale in R. This was first carried out without pooling the temporal replicates to evaluate the temporal stability of the population structure. Then, all temporal replicates were pooled, allowing for a spatial assessment of population structure. To investigate the temporal stability and spatial structure in more detail, we also performed a test for isolation by distance and a hierarchical analysis of molecular variance (AMOVA) in ARLEQUIN 3.0 (Excoffier *et al.* 2005) by partitioning the genetic variability into a within-site (F_{SC}) and between-site (F_{CT}) component. Third, for comparison, we also assessed population structure with a Bayesian Markov chain Monte Carlo (MCMC) assignment method based on multilocus genotypes, implemented in STRUCTURE 2.3.3. (Pritchard *et al.* 2000). The most likely structure was calculated assuming admixture and correlated allele frequencies. Each run, considering

population structure according to a specific number of groups ($1 \leq K \leq 15$), consisted of three chains of 10^5 MCMC replicates, initiated by 10^4 burn-in steps. Finally, to quantify migration in the study area, we used a Bayesian clustering method to identify first-generation migrants, implemented in GENECLASS 2.0 (Piry *et al.* 2004). For each individual, the probability was calculated to belong to the pool of microsatellite-based multilocus genotypes from the site where it was captured. Probability computation was based on Monte Carlo resampling with 10^3 simulated individuals (Rannala & Mountain 1997), and significance was evaluated at the 5% level.

Lateral plate number, Eda allele frequencies and migration. First, we investigated the distribution of plate number and the *Eda* allele frequencies at every site. Second, an ANOVA was used to analyse the occurrence of the Eda^L allele (scored as 0, 1 or 2 alleles per individual). Factors in this model included site as a fixed factor and season nested in site as a random factor. Third, correlations between distance to the coast, average plate number and the Eda^L frequency were tested. Finally, for each site, we investigated the association between plate number and *Eda* on the one hand and migration on the other hand by (i) comparing the probability of migration (see above) among *Eda* genotypes; (ii) testing the correlation between the probability of migration and plate number; and (iii) comparing plate number between putative migrants and residents.

Signatures of selection within populations. Stickleback is a short-lived species, and the generation time of the investigated populations is presumably annual. Therefore, most individuals within a given season can be assigned to a specific life stage. Spring samples mostly contain adults of 1 year old. Summer samples mostly contain juveniles while adults become scarce, and samples in fall almost exclusively contain subadults. Life stage-specific *Eda* allele frequencies hence can be quantified, and local shifts in these frequencies might be indicative for an episode of local selection at the *Eda* gene. If so, it is expected that such shifts are correlated with the local environment. To test this expectation, we tested the correlation between habitat characteristics and the difference in Eda^L frequency between (i) juveniles captured in summer vs. adults captured in the preceding spring; (ii) subadults captured in fall vs. juveniles captured in the preceding summer; and (iii) adults captured in spring vs. subadults captured in the preceding fall. Because these metrics reflect a life stage-specific differential fitness advantage at the *Eda* gene in terms of (i) reproductive output, (ii) juvenile survival and (iii) subadult overwinter survival, we further refer to these indices as Δ*Eda*[reproductive output], Δ*Eda*[juvenile survival] and Δ*Eda*[overwinter survival], respectively. For each of these indices, positive values suggest a fitness advantage for the Eda^L allele, while negative values suggest a fitness advantage for the Eda^C allele. Pearson correlations between each index and the aforementioned habitat characteristics (mean values of the two subsequent seasons) were calculated and tested for significance. Distance to the coast was included as well, to account for any unmeasured factor correlated with a declining influence of the coastal environment. Explanatory variables with significant or nearly significant effects were included in multiple regressions. Model fit was compared based on the AIC_C criterion for small sample size. Because Δ*Eda*[reproductive output], Δ*Eda*[juvenile survival] and Δ*Eda*[overwinter survival] might not exclusively reflect differential reproductive output or mortality, but can also be influenced by migration, all computations were done with and without first-generation migrants (see above).

To test for additional indications for selection at the *Eda* gene, we calculated for each site the index Δ*Eda*[reproductive investment], that is the difference in Eda^L frequency between individuals that did and did not visibly invested in reproduction (see above). We expected this index to correlate with Δ*Eda*[reproductive output], as a shift in Eda^L frequency from one generation (adults in spring) to the next (juveniles in summer) should already be visible in the fraction of adults investing in reproduction. Furthermore, we also compared the three *Eda* genotypes for differences in body size at the juvenile (summer), subadult (fall) and adult (spring) stage, to test for a potential growth advantage associated with the *Eda* gene. For juveniles, this test result might as well reflect an age advantage arising from faster maturation of the parents. This analysis was performed with general linear models (one per life stage) with standard length as the dependent variable and site, *Eda* genotype and the site by *Eda* genotype interaction as independent variables. For adults in spring, sex was also included in the model. Sampling year was included as a random block factor.

Selection vs. gene flow. Four methods (I-IV) were used to quantify the relative contribution of selection and gene flow to the differentiation in plate number and at the *Eda* gene. (I) Outlier detection tests implemented in LOSITAN (Antao *et al.* 2008) and BAYESCAN (Foll & Gaggiotti 2008) were used to evaluate whether the global F_{ST} at the *Eda* locus classified as a non-neutral outlier (indicative of selection) as compared to the microsatellite markers. A false discovery rate of 0.05 was applied for both methods. (II) Likewise, a F_{ST} –P_{ST} approach (Raeymaekers *et al.* 2007; Leinonen *et al.* 2008) was used to

investigate whether the global differentiation in plate number (P_{ST}[plates]) exceeded the global neutral genetic differentiation as quantified with microsatellite markers. (III) Correlation analysis and multiple regressions were used to explain the interpopulation variation in average plate number and the Eda^L frequency. Explanatory variables included the aforementioned habitat characteristics (mean values over the entire period). Population-specific F_{ST} (as calculated in GESTE; Foll & Gaggiotti 2006) and allelic richness were included as well, to evaluate whether phenotypic variation (plate number) and the underlying genetic variation (*Eda*) are associated with neutral genetic processes. As above, explanatory variables with significant or nearly significant simple effects were included in multiple regressions, of which the model fit was compared based on the AIC_C criterion for small sample size. (IV) Simple and partial Mantel tests on population pairs were used to identify the determinants of pairwise differentiation at the *Eda* locus (F_{ST}[*Eda*]), as well as pairwise differentiation in plate number (P_{ST}[plates]). Explanatory variables included pairwise differentiation (F_{ST}) at neutral markers as well as pairwise Euclidean distances based on habitat characteristics. For each of these analyses (I–IV), one population (L10) was excluded as its extremely low genetic diversity suggested that it might have been introduced or gone through a recent bottleneck, and hence, might be not in selection–migration–drift balance with the surrounding populations.

Meta-analysis

A meta-analysis of the relative strength of selection targeting the *Eda* locus vs. homogenizing gene flow was performed to compare our results on the lowland populations with five other studies (Raeymaekers *et al.* 2007; Mäkinen *et al.* 2008; Van Dongen *et al.* 2009; DeFaveri *et al.* 2011; DeFaveri & Merilä 2013). These studies were performed on natural populations of three-spined sticklebacks sampled across various spatial scales (Table 2). Populations were either selected along a salinity cline, or chosen from a set of discrete habitats with different salinities (Table 2). All data sets included plate number (except for the study by DeFaveri *et al.* 2011), neutral genetic markers and markers linked to the *Eda* gene (*STN365*, *STN380* or *STN381*). On one occasion, only plate number and neutral genetic data have been published (Van Dongen *et al.* 2009), but *Eda* genotypes of the same populations were available.

Each data set was analysed in parallel with the lowland data set according to analyses I to IV, as outlined above. The results of analysis I (outlier tests with LOSITAN and BAYESCAN) and analysis II (F_{ST}–P_{ST} comparisons) were summarized across studies with a linear regression analysis regressing global values for F_{ST}[*Eda*] and P_{ST}[plates] on global values for neutral F_{ST}. Analysis III consisted of testing correlations between neutral genetic diversity on the one hand and average plate number and the Eda^L frequency on the other hand. For analysis IV, correlations between pairwise differentiation at neutral genetic markers (pairwise F_{ST}), pairwise differentiation at the *Eda* locus (pairwise F_{ST}[*Eda*]) and pairwise differentiation in plate number were computed. For two data sets that also contained detailed salinity data (i.e. the current study and the Baltic Sea study by DeFaveri & Merilä 2013), correlations with salinity were calculated as well.

Results

Habitat structure

Abiotic and biotic characteristics, averaged over the period 2008–2010, are presented in Table 1. Overall, sites differed significantly in salinity ($F_{14,26} = 8.50$; $P < 0.0001$), pH ($F_{14,26} = 3.97$; $P = 0.0012$) and water depth ($F_{13,25} = 8.19$; $P < 0.0001$). Local fluctuations in environmental conditions were small (in particular for salinity) or could be attributed to seasonal effects (such as rainfall) affecting the entire region. Salinity ranged between 0.18 and 2.38 psu and declined with distance from the coast ($R = -0.59$; $P = 0.0201$). Values for pH were correlated with salinity ($R = 0.69$; $P = 0.0043$). Other habitat characteristics did not significantly correlate with salinity, and only water depth was significantly correlated with distance to the coast as well ($R = 0.66$; $P = 0.0104$). Six sites had brackish water (L01–L06; equivalent of conductivity values >1000 μS/cm) and clustered along the first axis of a principal component analysis (Fig. 1). The remaining sites had freshwater (conductivity values <1000 μS/cm). These sites had more heterogeneous PC values, reflecting either a high density of macro-invertebrate predators (L07, L11), a high density of nine-spined sticklebacks (L12, L13, U01) or a high turbidity (U02). Predatory fishes included eel (*Anguilla anguilla* L.; observed occasionally at L08 and L12) and perch (*Perca fluviatilis* L.; common at L06).

Population genetic structure

Genetic diversity, quantified as allelic richness, declined with distance from the coast ($R = -0.75$; $P = 0.0021$; Fig. 2). AMOVA revealed significant genetic structure among sites ($F_{CT} = 0.046$; $P < 0.0001$) and, albeit much weaker, among temporal replicates within sites ($F_{SC} = 0.003$; $P < 0.0001$; Fig. S1, Supporting information). A CMDS plot based on pairwise F_{ST} revealed clustering of the sites near the coast, including those

Table 2 Summary of six studies assessing the divergence in lateral plate number and the *Eda* locus in natural stickleback populations. Populations were sampled across various spatial scales, and either selected along a salinity cline, or chosen from a set of discrete habitats with different salinities

			Lateral plates		Neutral markers			*Eda*-linked markers			$R_{Pearson}$		R_{Mantel}			
Study (N_{pop}/N_{ind})	Spatial scale	Salinity (range)	MPN (range)	P_{ST}	Type (N_{LOC})	H_O (range)	F_{ST} (95% CI)	Marker	H_O [*Eda*]	F_{ST}[*Eda*]	AR vs. MPN	AR vs. Eda^L	F_{ST} vs. F_{ST}[*Eda*]	F_{ST} vs. P_{ST}	F_{ST}[*Eda*] vs. P_{ST}	References
Lowlands (14/2320)	78 km	Fresh-brackish (discrete)	5.85–19.26	0.15	μsats (15)	0.62–0.75	0.046 (0.041–0.052)	STN 380	0.478	$0.115^{**/NS}$	0.62*	−0.72**	0.42**	0.28°	0.83***	Current study
Baltic (14/480)	~1000 km	Fresh-salt (clinal)	14.61–25	0.22	μsats (20)	0.66–0.74	0.006 (0.004–0.009)	STN 381	0.367	0.101***/**	0.25NS	-0.22^{NS}	0.32°	0.16^{NS}	0.85***	DeFaveri & Merilä (2013)
LU2002 (8/400)	~200 km	Fresh-brackish (discrete)	4.97–19.58	0.41	μsats (15)	0.63–0.78	0.096 (0.079–0.111)	STN 381	0.277	0.382**/**	0.75*	−0.83*	0.07^{NS}	0.05^{NS}	0.93*	Raeymaekers *et al.* (2007)
LU2004 (15/747)	~300 km	Fresh-brackish (discrete)	4.98–19.23	0.37	μsats (15)	0.61–0.82	0.083 (0.073–0.095)	STN 380	0.338	0.351***/***	0.79***	−0.87***	-0.04^{NS}	0.02^{NS}	0.89***	Barrett *et al.* (2009),
								STN 381	0.362	0.406***/***		−0.83***	-0.07^{NS}		0.91***	Van Dongen *et al.* (2009)
Europe (7/168)	Continental	Fresh-salt (discrete)	4.27–32.54	0.62	μsats (102)	0.52–0.74	0.166 (0.154–0.178)	STN 365	0.64	0.653***/***	0.27^{NS}	—	0.08^{NS}	0.36^{NS}	0.71**	Mäkinen *et al.* (2008)
								STN 380	0.607	0.685***/***		—	0.14^{NS}		0.71**	
								STN 381	0.553	0.856***/***		-0.06^{NS}	0.15^{NS}		0.71**	
Global (12/288)	Global	Fresh-salt (discrete)	—	—	μsats (23)	0.60—0.84	0.149 (0.123–0.178)	STN 365	0.67	0.405***/***	—	—	0.13^{NS}	—	—	DeFaveri *et al.* (2011)

N_{POP}, number of populations; N_{IND}, number of individuals; MPN, mean plate number; P_{ST}, proportion of among-population phenotypic variance; N_{LOC}, number of loci; H_O and H_O[*Eda*], average observed heterozygosity at neutral markers and the *Eda* locus; F_{ST} and F_{ST}[*Eda*], standardized allelic variance at neutral markers and at the *Eda* locus; AR, allelic richness at neutral markers; Eda^L, frequency of the *Eda* allele for the low morph,, $R_{Pearson}$, Pearson correlation; R_{Mantel}, Mantel correlation among pairwise metrics of differentiation.

P-values ($^{NS}P > 0.10$; $^{\circ}P < 0.10$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$) are provided for $R_{Pearson}$ and R_{Mantel}, as well as for F_{ST}[*Eda*] (before the dash: test with LOSITAN; after the dash: test with BAYESCAN).

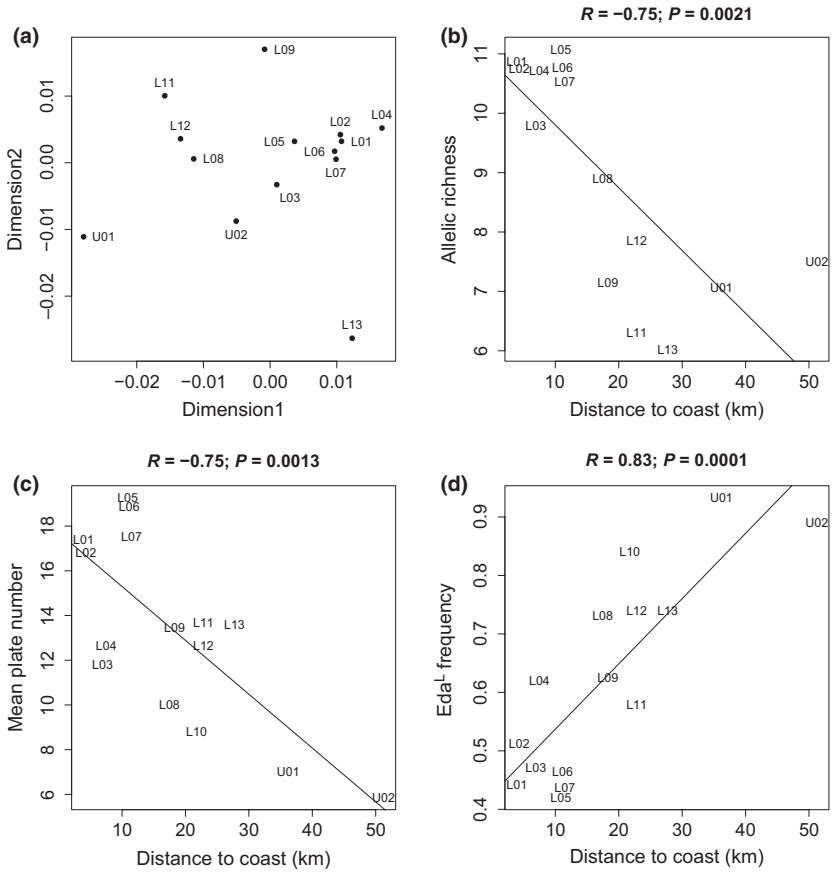

Fig. 2 (a) Classical multidimensional scaling plot of neutral population genetic structure based on pairwise F_{ST}. Correlation between distance to the coast and (b) allelic richness at neutral markers, (c) the average number of lateral plates and (d) the Eda^L frequency. Population codes as in Table 1. Population L10 is excluded from plots a and b.

that were geographically distant (Fig. 2). Accordingly, the test for isolation by distance was not significant (geographical distance vs. neutral pairwise F_{ST}: $R = 0.11$; $P = 0.29$). Bayesian analysis with STRUCTURE suggested an optimal structure with seven clusters: two mixing 'coastal' clusters containing individuals from populations L01 to L07, a cluster containing populations L08, L11 and L12, a cluster containing populations U01 and U02, and three clusters containing a single population (L09, L10, L13). The analyses of first-generation migrants revealed that the probability of migration did not correlate with distance to the coast ($R = -0.28$; $P = 0.31$) or salinity ($R = 0.24$; $P = 0.38$).

Lateral plate number, Eda allele frequencies and migration

All sites were polymorphic for plate number (Fig. 1a). In line with previous studies (Raeymaekers *et al.* 2007; Lucek *et al.* 2012), the contingency between plate morph (completely plated – partially plated – low-plated) and indel-based *Eda* genotype (CC: homozygous for Eda^C; CL: heterozygous; LL: homozygous for Eda^L) was good, albeit not perfect (Fig. 1a; see Lucek *et al.* (2012) for potential causes). Differences in the Eda^L frequency between sites as well as fluctuations between seasons are shown in Fig. S2 (Supporting information). ANOVA revealed that the occurrence of the Eda^L allele (0, 1 or 2 times per individual) varied significantly between sites ($F_{14,2515} = 28.73$, $P < 0.0001$), as well as between seasons within sites ($F_{50,2515} = 1.62$, $P = 0.004$). At the landscape level, we observed that average plate number declined with distance from the coast ($R = -0.75$; $P = 0.0013$; Fig. 2), whereas the Eda^L frequency increased ($R = 0.83$; $P = 0.0001$; Fig. 2). The analysis of first-generation migrants across and within sites identified no association between *Eda* genotype and the probability of migration, no correlation between the probability of migration and plate number, and no difference in plate number between putative residents and migrants (results not shown).

Signatures of selection within populations

Δ*Eda*[reproductive output], quantifying the difference in Eda^L frequency between juveniles captured in summer and the corresponding adults captured in the preceding spring, mostly indicated a higher Eda^L frequency in juveniles than in adults in freshwater and a lower Eda^L frequency in juveniles than in adults in brackish (and

more alkaline) water. As a result, this index was negatively correlated with salinity ($R = -0.61$; $P = 0.0269$; Fig. 3) and pH ($R = -0.56$; $P = 0.0471$). Multiple regression followed by AICc-based model selection failed to determine whether either salinity or pH was the best predictor of Δ*Eda*[reproductive output] (i.e. $\Delta AIC_C < 2$). Δ*Eda*[reproductive output] was also positively correlated with Δ*Eda*[reproductive investment], that is the difference in Eda^L frequency in spring between individuals that did and did not visibly invested in reproduction ($R = 0.63$; $P = 0.0208$; Fig. 3). This suggests that the shift in Eda^L frequency from one generation to the next was indeed caused by differential investment in reproduction in spring. Neither Δ*Eda*[reproductive output] nor Δ*Eda*[reproductive investment] were correlated with distance to the coast (Fig. 3) or any other habitat characteristic.

Δ*Eda*[juvenile survival], quantifying the difference in Eda^L frequency between subadults in fall and juveniles in the preceding summer, did not correlate with any habitat characteristic or geographical variable. Δ*Eda*[overwinter survival], quantifying the difference in Eda^L frequency between adults in spring and subadults in the preceding fall, was positively correlated with pH ($R = 0.61$; $P = 0.0456$; Fig. 3), but negatively correlated with distance to the coast ($R = -0.64$; $P = 0.0333$; Fig. 3). Multiple regression followed by AICc-based model selection failed to determine whether either distance to the coast or pH was the best predictor of this index (i.e. $\Delta AIC_C < 2$).

In summary, shifts in Eda^L frequencies between adults in spring and juveniles in the subsequent summer were negatively correlated with salinity and pH and positively correlated with differential reproductive investment. Shifts in Eda^L frequencies between subadults in fall and adults in the subsequent spring were positively correlated with pH (and were hence in the opposite direction), but were also correlated with distance to the coast. All of the above results were confirmed when the analyses were repeated without first-generation migrants (results not shown). Furthermore, we did neither observe significant effects of *Eda* genotype, nor an *Eda* genotype by site interaction effect when comparing body size at the juvenile (summer), subadult (fall) or adult stage (spring; Fig. S3, Supporting information).

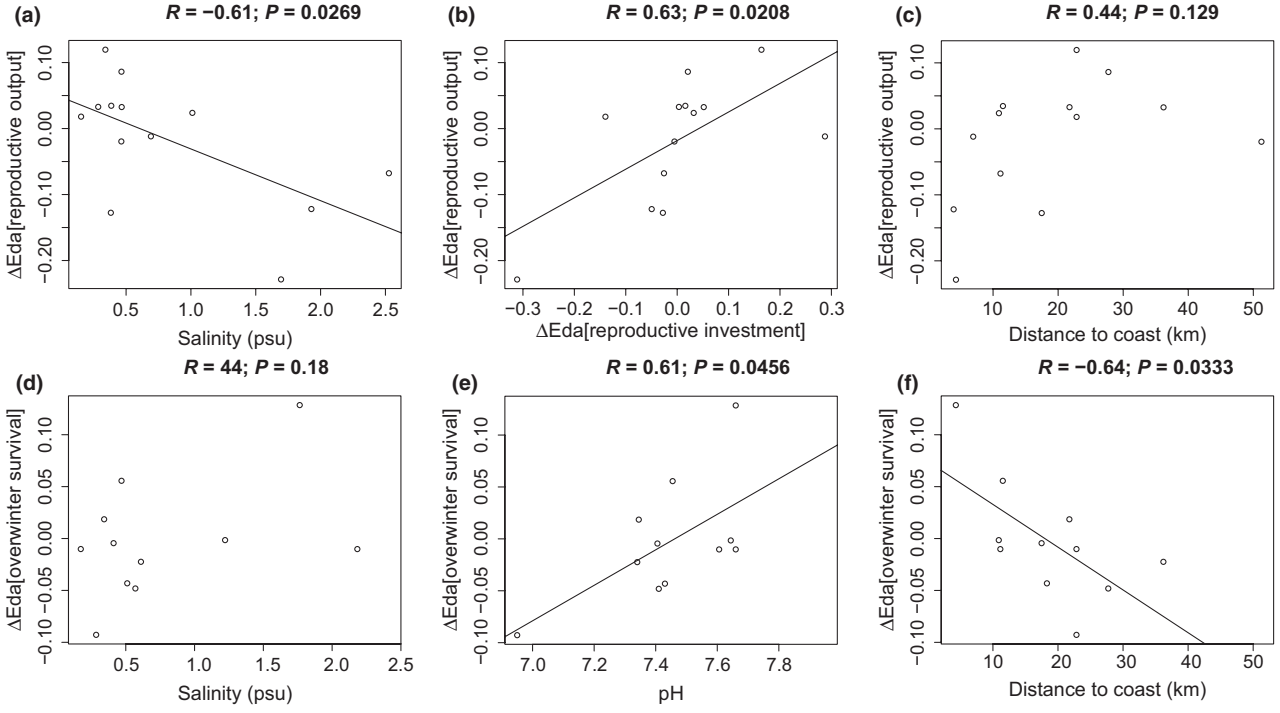

Fig. 3 Correlations between habitat characteristics, distance to the coast, Δ*Eda*[reproductive output], Δ*Eda*[reproductive investment] and Δ*Eda*[overwinter survival]. Δ*Eda*[reproductive output] is the difference in Eda^L frequency between juveniles captured in summer and the corresponding adults captured in the preceding spring. Δ*Eda*[reproductive investment] is the difference in Eda^L frequency in spring between individuals that did and did not visibly invest in reproduction. Δ*Eda*[overwinter survival] is the difference in Eda^L frequency between adults captured in spring vs. subadults captured in the preceding fall. (a) Salinity vs. Δ*Eda*[reproductive output]; (b) Δ*Eda*[reproductive investment] vs. Δ*Eda*[reproductive output]; (c) Distance to the coast vs. Δ*Eda*[reproductive output]; (d) Salinity vs. Δ*Eda*[overwinter survival]; (e) pH vs. Δ*Eda*[overwinter survival]; (f) Distance to the coast vs. Δ*Eda*[overwinter survival].

Selection vs. gene flow

While the LOSITAN analysis classified the *Eda*-linked marker STN380 as a locus putatively under divergent selection (F_{ST}[*Eda*] = 0.115; P = 0.008), BAYESCAN did not (P = 0.9642). The global P_{ST} value (P_{ST} = 0.15) exceeded neutral F_{ST}(F_{ST} = 0.046, 95% CI = 0.042–0.052), suggesting a contribution of divergent selection to phenotypic differentiation.

Among all habitat characteristics, only salinity was marginally correlated with Eda^L frequency and mean plate number. The Eda^L frequency decreased with salinity (R = −0.50, P = 0.0549, Fig. 4a), while mean plate number increased with salinity (R = 0.52, P = 0.0484, Fig. 4b). However, in both cases, the correlation with allelic richness was stronger (Eda^L frequency: R = −0.72, P = 0.0035, Fig. 4c; mean plate number: R = 0.62, P = 0.0190, Fig. 4d), as was the correlations with population-specific F_{ST}: (R = 0.65, P = 0.0126; mean plate number: R = −0.54, P = 0.0459). Multiple regressions followed by AICc-based model selection confirmed that AR and F_{ST} were better predictors of the *Eda* allele frequency than salinity (i.e. all $\Delta AIC_C > 2$; results not shown). This was also the case for plate number, except for F_{ST} vs. salinity, showing about equal effects (i.e. $\Delta AIC_C < 2$).

A similar pattern emerged from the analysis of pairwise F_{ST}[*Eda*] and pairwise P_{ST} for plate number. Pairwise F_{ST}[*Eda*] was not correlated with pairwise differential salinity (R = 0.003, P = 0.43; Fig. 4e), but significantly increased with neutral pairwise F_{ST} (R = 0.42; P = 0.009; Fig. 4g). Likewise, pairwise P_{ST} for plate number did not increase with pairwise differential salinity (R = 0.079, P = 0.27; Fig. 4f), but was marginally correlated with neutral pairwise F_{ST} (R = 0.28; P = 0.064; Fig. 4h). Partial Mantel tests revealed that the increase in pairwise F_{ST}[*Eda*] with neutral pairwise F_{ST} remained significant after correction for any habitat characteristic (results not shown). Figure 4G, H shows that P_{ST} for plate number and F_{ST}[*Eda*] remained low in the face of high gene flow (neutral F_{ST} < 0.02; this included all population pairs from site L01 to L07).

Meta-analysis

The LOSITAN and BAYESCAN outlier analyses always classified the *Eda*-linked markers as loci putatively under divergent selection, except for the lowland data set (i.e. the current study) for the analysis with BAYESCAN (Table 2; Fig. S4, Supporting information). Overall P_{ST} for plate number always significantly exceeded the

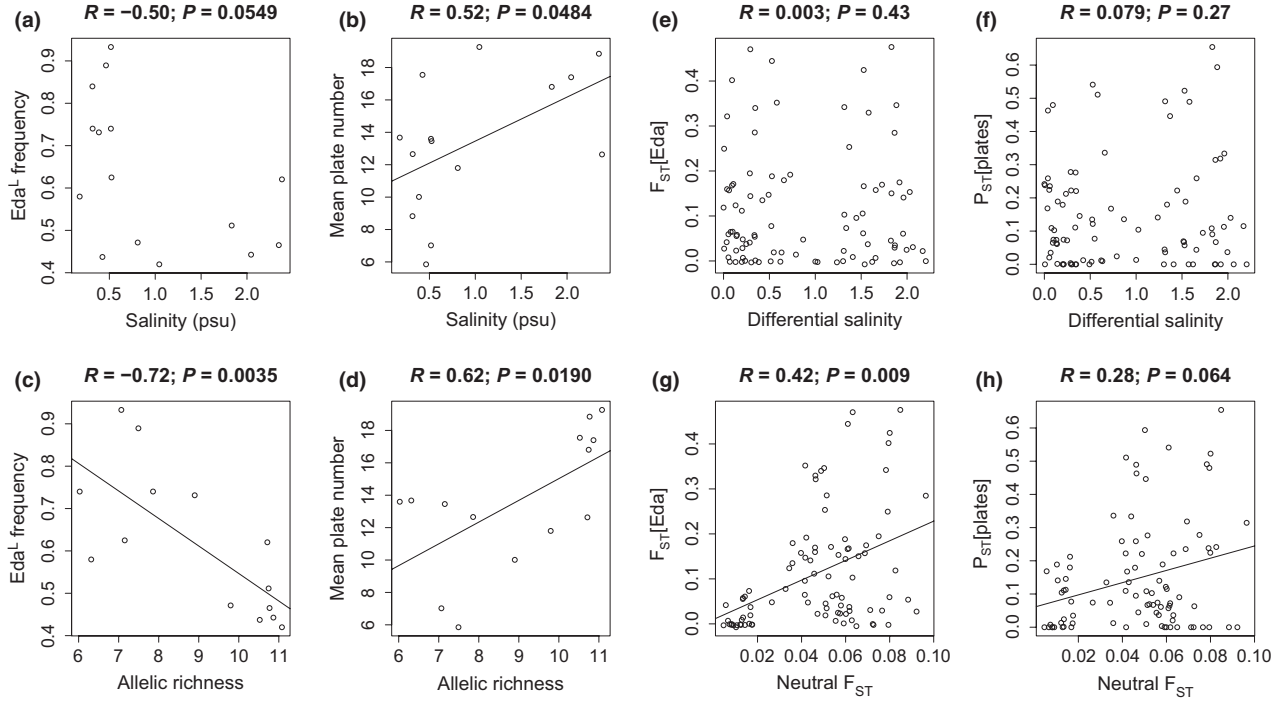

Fig. 4 Associations between neutral genetic diversity, salinity, the *Eda* locus and plate number in polymorphic stickleback populations from the Belgian-Dutch lowlands. (a) Eda^L frequency vs. salinity. (b) Mean plate number vs. salinity. (c) Eda^L frequency vs. allelic richness at neutral markers. (d) Mean plate number vs. allelic richness at neutral markers. (e) Pairwise F_{ST}[*Eda*] vs. pairwise differential salinity. (f) Pairwise P_{ST} for lateral plate number vs. pairwise differential salinity. (g) Pairwise F_{ST}[*Eda*] vs. neutral pairwise F_{ST}. (h) Pairwise P_{ST} for lateral plate number vs. neutral pairwise F_{ST}.

neutral genetic differentiation (Table 2; distribution of plate number in each study system is provided in Fig. S5, Supporting information). The relationship between overall neutral F_{ST} and overall F_{ST}[*Eda*] across studies was positive ($R = 0.93$, $P = 0.0078$, Fig. 5). This was also the case for the relationship between overall neutral F_{ST} and overall P_{ST} for plate number ($R = 0.93$, $P = 0.0198$, Fig. 5). The current study deviated from these overall trends by having lower overall F_{ST}[*Eda*] and P_{ST} than predicted by the overall neutral F_{ST}, hence having the lowest ratio of F_{ST}[*Eda*] vs. neutral F_{ST} and the lowest ratio of P_{ST} vs. neutral F_{ST}.

The correlation between neutral genetic diversity and mean plate number was positive or nonsignificant, while the correlation between neutral genetic diversity and the Eda^{L} frequency was negative or nonsignificant (Table 2; Fig. S6, Supporting information). Correlations between neutral pairwise F_{ST} and pairwise F_{ST}[*Eda*] and pairwise P_{ST} for plate number were always nonsignificant, except for the current study where the correlation between neutral F_{ST} and F_{ST}[Eda] was positive (Table 2; Fig. S7, Supporting information). Interestingly, the Baltic system was characterized by F_{ST}[*Eda*] and P_{ST} values similar to the current study system, despite stronger neutral gene flow (Fig. 5). Accordingly, correlations between salinity and pairwise F_{ST}[*Eda*] or pairwise P_{ST} for plate number in the Baltic system (salinity vs. F_{ST}[*Eda*]: $R = 0.30$, $P = 0.09$; salinity vs. P_{ST}: $R = 0.28$, $P = 0.01$) were stronger than in the current study (salinity vs. F_{ST}[*Eda*]: $R = 0.003$, $P = 0.43$; salinity vs. P_{ST}: $R = 0.08$, $P = 0.27$; Fig. 6).

Discussion

The aim of this study was to elucidate the relative contribution of divergent selection vs. homogenizing gene flow to allelic variation at a gene with major effects on an ecologically relevant trait. A classical example of such a gene is the Ectodysplasin (*Eda*) gene, the major gene controlling lateral plate phenotype in three-spined sticklebacks. We tracked the allele frequencies at this locus as well as at a set of neutral loci in 15 populations polymorphic for lateral plate number. Assessing the effect of divergent selection and gene flow at both the phenotypic and genetic level was relevant, because *Eda* accounts for more than 75% of the variation in the phenotype (Colosimo *et al.* 2004). Various studies have investigated adaptive polymorphisms in relationship to local selection and dispersal or gene flow. Examples include the *PGI* gene in *Colias* and *Melitaea* butterflies (Watt *et al.* 2003; Haag *et al.* 2005), haemoglobin in yellow-billed pintails (McCracken *et al.* 2009), MHC class IIb genes in lake and stream three-spined sticklebacks (Wegner *et al.* 2003), the rhodopsin gene in sand goby (Larmuseau *et al.* 2009) and pigmentation genes in pocket mice (Hoekstra *et al.* 2004).

The relative strength of divergent selection vs. opposing gene flow provides a general explanation for why populations may or may not diverge (Levene 1953; Endler 1973), and this has also been considered in sticklebacks (Hagen 1967; Bell 1982; Moore *et al.* 2007; Hendry *et al.* 2009). As such, it represents an important element in the ongoing discussion on the degree of parallelism in stickleback evolution (Berner *et al.* 2010; Jones *et al.* 2012; Ravinet *et al.* 2013). Below we discuss indications in our study system for divergent selection on the one hand and for the homogenizing effect of gene flow on the other hand.

Indications for divergent selection

Several experiments have revealed a differential selective advantage for the different plate morphs as well as for the allelic variants of the *Eda* gene. These differences were observed in response to either the abiotic environment (salinity) or to predation. With respect to salinity, Heuts (1947) observed that completely plated sticklebacks hatch more successfully in saltwater, whereas

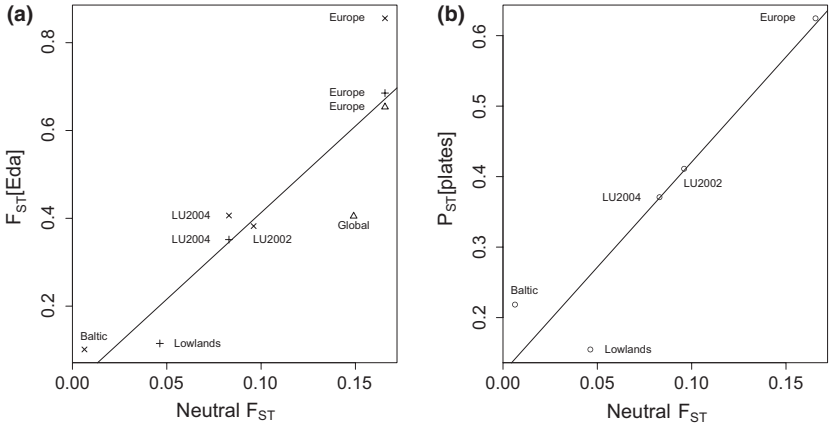

Fig. 5 Meta-analysis across six population genetic studies of three-spined stickleback. (a) Relationship between overall neutral F_{ST} and overall F_{ST}[*Eda*]. The symbols indicate the different *Eda*-linked markers (Δ, STN365; +, STN380; ×, STN381). (b) Relationship between overall neutral F_{ST} and overall P_{ST} for lateral plate number. Study systems are labelled as in Table 2.

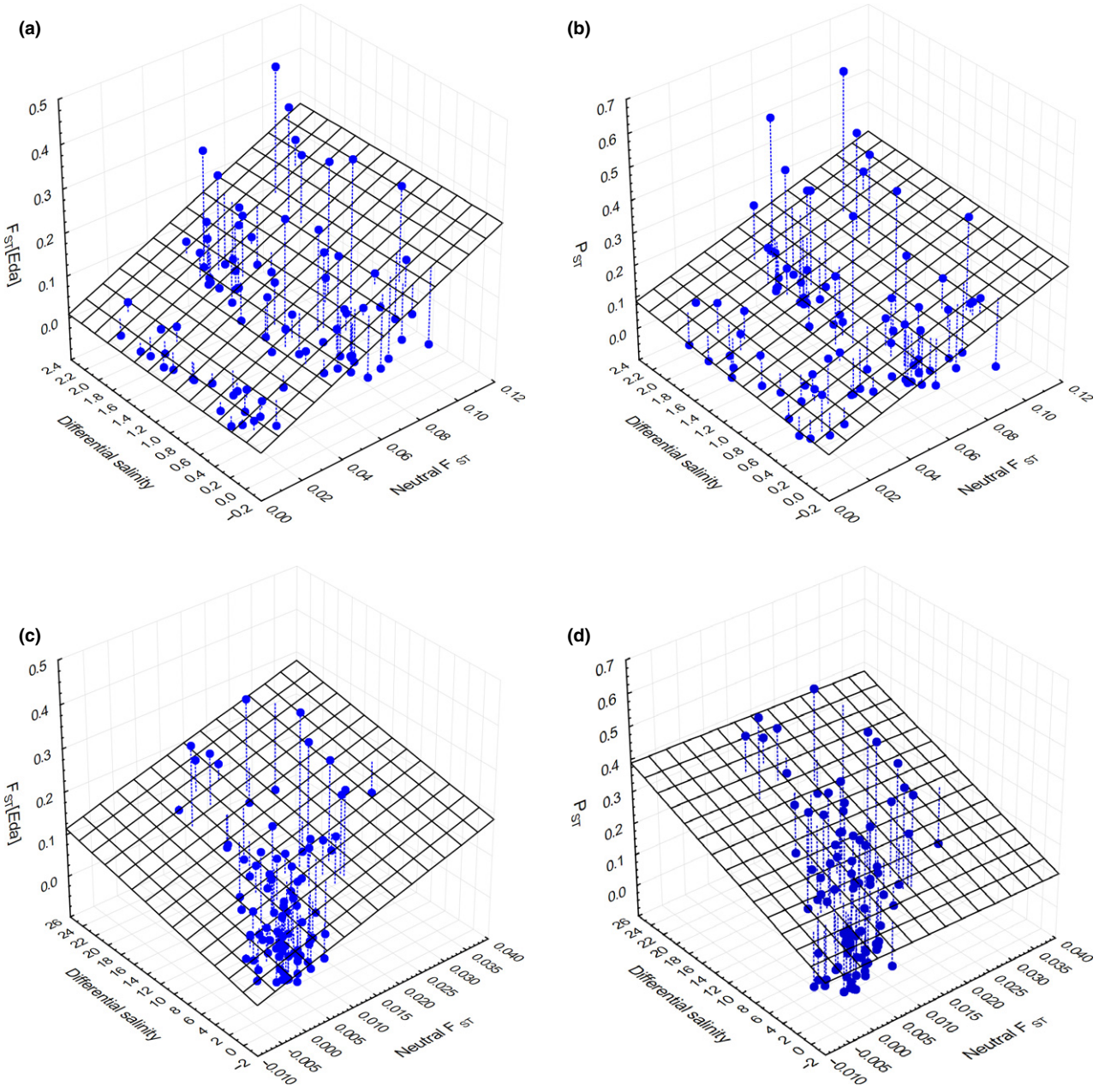

Fig. 6 Comparison of the lowland study system from Belgium and the Netherlands (current study) with the Baltic study system (DeFaveri & Merilä 2013). (a) Pairwise F_{ST}[*Eda*] vs. neutral pairwise F_{ST} and pairwise differential salinity in the current study. (b) Pairwise P_{ST} for lateral plate number vs. neutral pairwise F_{ST} and pairwise differential salinity in the current study. (c) Pairwise F_{ST} [*Eda*] vs. neutral pairwise F_{ST} and pairwise differential salinity in the Baltic study system. (d) Pairwise P_{ST} for lateral plate number vs. neutral pairwise F_{ST} and pairwise differential salinity in the Baltic study system.

low-plated sticklebacks hatch more successfully in freshwater. Marchinko & Schluter (2007) found that low-plated sticklebacks grow faster in freshwater than completely plated sticklebacks, whereas there was no such difference in saltwater. Barrett *et al.* (2008) found that fish carrying the Eda^{L} allele gain a growth advantage in freshwater, leading to higher overwinter survival and reproductive success. Barrett *et al.* (2009) found that this growth advantage is due to a pleiotropic effect of the *Eda* gene on armour and growth. With respect to predation, Reimchen (2000) demonstrated that completely plated sticklebacks have an advantage expressed as increased protection and escape opportunities. Leinonen *et al.* (2011) confirmed Reimchen's hypothesis that this advantage is habitat dependent, as in the presence of a refuge low-plated sticklebacks are

more probably to escape from predatory fish than completely plated sticklebacks. Finally, Marchinko (2009) observed increased survival for individuals carrying the Eda^L allele in response to macro-invertebrate predation, whereas Zeller *et al.* (2012a) did not.

In this study, a differential selective advantage at the *Eda* gene was suggested when comparing subsequent generations. The *Eda* allele frequencies of juveniles in summer vs. representatives of the parental generation in the preceding spring showed a shift towards lower plate number in freshwater and a shift towards higher plate number in brackish (and more alkine) water. This shift from one generation to the next was significantly correlated with the differences in *Eda* allele frequency between adults who visibly invested in reproduction vs. adults that did not. Therefore, this pattern is probably indicative for greater (or earlier) reproductive success of individuals carrying the Eda^L allele in freshwater (i.e. similar to findings in Barrett *et al.* 2008) and of individuals carrying the Eda^C allele in brackish water. However, we cannot attribute these differences in reproductive success to a growth advantage, as we did not observe significant differences in body size between the *Eda* genotypes at any life stage. We also cannot confirm a role for predation, as neither *Eda* allele frequencies nor changes in *Eda* allele frequencies were correlated with the density of macro-invertebrate predators.

Indications for the homogenizing effect of gene flow

Despite the indications for divergent selection in response to salinity levels from one generation to the next, salinity was only marginally correlated with the variation at the *Eda* locus and variation in plate number in our field survey. Likewise, pairwise differentiation at the *Eda* gene and pairwise P_{ST} for plate number did not correlate with pairwise differential salinity. The main reason for this lack of correlation was that the populations at some of the brackish sites still included a considerable fraction of low-plated individuals (e.g. L04), while populations at some of the freshwater sites still included a considerable fraction of completely plated individuals (e.g. L07). Relatively weak correlations between plate number and salinity or conductivity have been reported in other studies (Hagen & Gilbertson 1972; DeFaveri & Merilä 2013). While this suggests that salinity might not be the main selective agent for plate number, we did not observe strong correlations with other environmental cues either. Admittedly, our environmental assessment was not exhaustive, and other factors influencing the selective advantage of *Eda* genotypes have been proposed, such as calcium concentrations (Giles 1983).

Remarkably, however, variation in plate number and allelic variation at the *Eda* locus was strongly correlated with neutral population structure. Neutral genetic diversity outperformed salinity as a predictor of *Eda* allele frequency and mean plate number. Pairwise differentiation at the *Eda* locus increased with pairwise neutral F_{ST} and remained low in contrasts between populations characterized by low F_{ST} values, indicating substantial gene flow. There are two potential explanations for these correlations. First, adaptive divergence at the *Eda* locus due to a strong environmental cue might constrain gene flow between divergent populations (i.e. isolation by adaptation; Funk *et al.* 2011). If so, we clearly failed to document such environmental cue. However, if present, it would probably be correlated with distance to the coast (indicative for the declining influence of the coastal environment), which was also a strong predictor of plate number and *Eda* allele frequency. Second, high gene flow might constrain adaptive divergence by homogenizing allele frequencies at the *Eda* locus. This possibility seems more likely. It is known that some completely plated stickleback populations are anadromous, performing spawning migrations from coastal to freshwater habitats after a substantial raise in water temperature in spring (Wootton 1976). Such behaviour has also been reported in our study area (Tudorache *et al.* 2007). At least part of our freshwater populations might hence be exposed to high levels of gene flow from such anadromous populations, counteracting selection by a new influx of maladaptive or subadaptive alleles. Another indication that dispersal and gene flow are substantial in the lowlands is the fast recolonization of ditches and streams after summer droughts (personal observation).

The adaptive significance of variation in lateral plate phenotypes has received much attention since the observation that lateral plates might be targeted by selection (Hagen & Gilbertson 1973). In contrast, the possibility of gene flow counteracting selection on lateral plate phenotypes has been rarely tested. In a study of clinal variation for the number of lateral plates within the low-plated morph across a stream gradient, Bell & Richkind (1981) concluded that gene flow must counter selection. Likewise, Baumgartner (1986) demonstrated that both selection and gene flow shape the distribution of plate morphs in two adjacent streams. Since the discovery of the major effect of the *Eda* gene on plate number (Colosimo *et al.* 2005), studies still focus on the role of selection, but genomic studies testing for signatures of selection at the *Eda* gene typically account for genome-wide differentiation, which is partially reflecting gene flow (e.g. Mäkinen *et al.* 2008; DeFaveri *et al.* 2011; DeFaveri & Merilä 2013). A meta-analysis on six such studies provided further support for the

homogenizing effect of gene flow in our study system. First, compared to the other stickleback systems, the signature of selection at *Eda* in this study was weak. Second, the lowland system was the only system where pairwise neutral F_{ST} correlated significantly with F_{ST}[*Eda*]. Comparison with the Baltic system (DeFaveri & Merilä 2013) revealed that divergence at the *Eda* locus is possible even in the face of stronger gene flow than in our study. The difference is probably due to a broader range of salinities or correlated environmental effects in the Baltic (saltwater to freshwater) than in this study (brackish to freshwater).

Limitations and alternative explanations

Anadromous migration by completely plated individuals provides a straightforward explanation for the persistence of individuals carrying the Eda^C allele in freshwater despite counteracting selection. However, one limitation of our study is that we did not find direct indications for migration load. Although this might be due to the lack of power to quantify migration using molecular markers, it is possible that anadromous populations are uncommon or only occur locally. Alternatively, it might be that anadromous and resident freshwater populations interbreed infrequently (Hagen 1967; Jones *et al.* 2006; Bell *et al.* 2010) and hence that substantial gene flow is only realized over several generations. The persistence of individuals carrying the Eda^L allele in brackish water despite counteracting selection suggests that gene flow is also important in the opposite direction. Gene flow from freshwater to resident brackish water and marine stickleback might again be facilitated by anadromous populations. Such introgression contributes to the retention of freshwater-adapted alleles in marine populations, which facilitates the evolutionary response to directional selection after freshwater colonization. This process is referred to as 'allelic recycling' (Schluter & Conte 2009; Bell & Aguirre 2013).

Alternative explanations of why populations might remain polymorphic for plate number include time since divergence, genetic constraints, relaxation of selection, environmental heterogeneity and balancing selection. Time since divergence explained the decline in plate number in Norwegian lakes which underwent isolation through gradual uplifting from sea level after deglaciation (Klepaker 1995). The position of the coastline in our study area has been shifting since the Holocene, and the current transgression was stabilized only 500 years ago as a result of human activities. Therefore, some of our study sites certainly escaped earlier from marine influence than others. Nevertheless, it is likely that all study sites have been interconnected for most of the time and therefore never experienced long periods of isolation. Moreover, it is known that completely plated populations can evolve towards almost 100% low platedness in less than two decades (Bell & Aguirre 2013).

Genetic constraints on the *Eda* gene making the gene less susceptible for selection than elsewhere, including different epistatic or pleiotropic fitness effects, are unlikely, given that monomorphic low-plated and monomorphic completely plated populations do occur further inland (Heuts 1947; Raeymaekers *et al.* 2008, 2009) and further north in the North Sea (Jones *et al.* 2006), respectively. Relaxation of selection is also an unlikely explanation for the maintenance of plate number polymorphism, given the indications for differential selective advantages at the *Eda* gene (see above). An important contribution of environmental heterogeneity is also unlikely as we found that habitat characteristics, in particular salinity, differed between sites and were relatively stable during the course of the study.

One possibility we cannot rule out at this stage is balancing selection. For instance, Barrett *et al.* (2008) observed indications for opposing selection at the *Eda* gene during different life stages, suggesting either that the *Eda* gene affects additional traits undergoing selection, or that linked loci are also affecting fitness. Genomic studies have pointed out that the *Eda* gene is contained within a large block of genes in ancient and stable linkage, increasing the potential for pleiotropic effects (Colosimo *et al.* 2005; Albert *et al.* 2008; Jones *et al.* 2012). For the current study, the shifts from fall to spring (i.e. Δ*Eda*[overwinter survival]) were opposite to those from spring to summer (i.e. Δ*Eda*[reproductive output]; Fig. 3), and this might in principle reflect opposing selection rather than a migration–selection balance. Nevertheless, such complex selection conditions are unlikely to generate the observed pattern of plate number declining with distance from the coast and its strong correlation with neutral genetic diversity.

Conclusion and implications

Our findings suggest that polymorphism and geographical variation for lateral plate number in the lowland stickleback populations from North-western Europe are maintained through a combination of divergent selection targeting the *Eda* gene and homogenizing gene flow. As a result, these populations were characterized by the lowest ratio of F_{ST} at the *Eda* gene vs. neutral F_{ST} in comparison with other stickleback systems, investigated across similar environmental gradients.

It has been hypothesized that the parallel evolution of freshwater phenotypes across the stickleback's distribution range is facilitated by standing variation in

marine populations, composed of genes which have been 'recycled' from freshwater to marine populations (Schluter & Conte 2009). Such a recycling mechanism requires substantial introgression between marine and freshwater populations, which is hindered by the high effective population size of the marine populations (Bell & Aguirre 2013). We here documented that substantial gene flow can act as one potential source of incomplete phenotypic parallelisms upon freshwater colonization. However, the presence of such high gene flow areas also implies that freshwater alleles might frequently exchange between freshwater and brackish water populations, which ultimately might speed up the recycling of freshwater alleles into marine populations.

Our study highlights how allele frequencies at ecologically relevant genes are shaped by the relative strength of selection and gene flow and shows that such genes do not always contribute much to local adaptation. This also implies that ecologically relevant genes are not always detectable with standard outlier tests, even when selection on these genes is detectable as an ongoing process.

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J.A.M.R. designed and coordinated the study, analysed the data and wrote the study. N.K. assisted with fieldwork and genotyping. M.H.D.L. assisted with genotyping and data analysis, B.H. genotyped the samples, L.D.M. and F.A.M.V. supervised the research project. All co-authors participated in the conception of the study, revised it critically and approved the final version.

Data accessibility

Morphological and genetic data, geographical distances, habitat characteristics, data sets for meta-analysis: Dryad doi: doi:10.5061/dryad.d6620.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Schematic overview of analyses performed on 15 populations of three-spined stickleback from the Belgian and Dutch lowlands. Numbers correspond to sample sizes per *Eda* genotype (CC/CL/LL), sampled between spring 2008 and fall 2010.

Fig. S1 Classical multidimensional scaling plot of neutral population genetic structure among 59 stickleback samples, based on pairwise F_{ST}. The samples belong to 14 populations that were sampled repeatedly (up to six times; see Table S1, Supporting information) between spring 2008 and fall 2010. Population L10 was excluded from the analysis.

Fig. S2 Frequency of the Eda^L allele in 15 polymorphic stickleback populations from Belgium and the Netherlands, sampled between spring 2008 and fall 2010. The A panel groups the data by season and uses different symbols and colours per site. A single sample from summer 2008 (site L07; see Table S1, Supporting information) was omitted. The B panel groups the data by site and uses different symbols per season. Vertical bars represent bootstrap-based standard errors.

Fig. S3 Body size as measured by standard length in (a) juveniles in summer (N = 793), (b) subadults in fall (N = 663) and (c) adults in spring (N = 484) in 15 stickleback populations from Belgium and the Netherlands. Blue, red and green symbols mark individuals that were homozygous for the Eda^C allele (CC), heterozygous (CL) or homozygous for the Eda^L allele (LL), respectively. Error bars represent 95% confidence limits. None of the differences between the three genotypes were significant.

Fig. S4 Outlier test results using Lositan (left) and Bayescan (right) in six stickleback systems. The markers linked to the *Eda* gene are labelled (STN365, STN380 or STN381) and were all detected as markers under divergent selection, except for the test with Bayescan in the current study (i.e. the lowland study system). A false discovery rate of 0.05 was applied for both methods. Study systems are labelled as in Table 2.

Fig. S5 Distribution of lateral plate number per locality in five stickleback systems. Localities are sorted from low to high average plate number. Horizontal bars represent standard errors. Study systems are labelled as in Table 2. The data represent one side of the fish only. For the data set from Europe (Mäkinen *et al.* 2008), the initial count considered both sides of the fish, and hence, the available numbers were divided by two. Keel plates were not included in the total count for the lowlands (current study), LU2002 (Raeymaekers *et al.* 2007) and LU2004 (Van Dongen *et al.* 2009) studies.

Fig. S6 Eda^L frequency vs. allelic richness at neutral markers (left), mean plate number vs. allelic richness at neutral markers (centre) and mean plate number vs. Eda^L frequency (right) in five stickleback systems. A linear regression line is drawn for significant relationships. Study systems are labelled as in Table 2.

Fig. S7 Pairwise F_{ST}[*Eda*] vs. neutral pairwise F_{ST} (left) and pairwise P_{ST} for lateral plate number vs. neutral pairwise F_{ST} (right) in six stickleback systems. As indicated by the linear regression line, only the correlation shown in the first plot was significant (pairwise F_{ST}[*Eda*] vs. neutral pairwise F_{ST} in the current study, i.e. the lowland study system). Study systems are labelled as in Table 2.